



NEW ENZYMATIC OXIDATION/OXYFUNCTIONALIZATION TECHNOLOGIES FOR ADDED VALUE BIO-BASED PRODUCTS

MAIN RESULTS

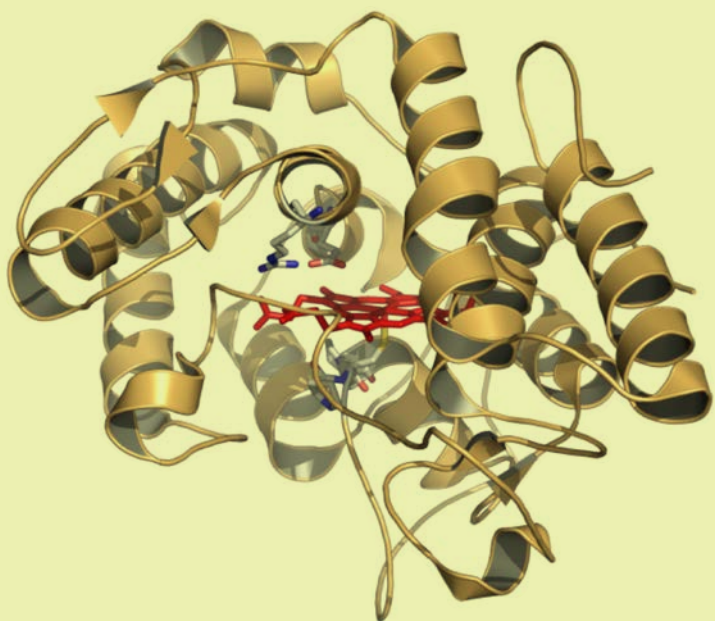
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PROJECT OBJECTIVE AND WORKFLOW

The overall objective of EnzOx2 is to develop new bio-chemical technologies based on oxidative enzymes, for the production of some added value compounds from biomass components to substitute others of petrochemical origin. The potential of oxidative enzymes in such biotransformations has been shown in previous projects, including different oxidation and oxyfunctionalization reactions catalyzed by fungal oxidoreductases (oxidases and peroxygenases) (1). To attain the above objective, the EnzOx2 project brings together three highly-specialized SMEs in the areas of fungal enzymes (**JenaBios**), 5-hydroxymethylfurfural (HMF) production (**AVA Biochem**) and chiral chemicals and active pharmaceutical ingredients (API) (**Chiracon**); two world-leading companies in the sectors of industrial enzymes (**Novozymes**) and flavour & fragrance ingredients (**Firmenich**); one technological centre dedicated to the plastics sector (**AIMPLAS**); and six research/academic partners with high expertise in oxidoreductase structure-function and engineering (**CIB** and **ICP**), their application in different biotransformations (**TUDresden** and **IRNAS**), the design and optimization of enzyme-based bioprocesses (**TUdelft**), and biocatalyst immobilization (together with other partners) and life cycle assessment analysis (**USC**).



In the above context, the EnzOx2 partners first took advantage from the largely unexploited **diversity** of oxidoreductases in fungi from special habitats, and oxidoreductase genes in sequenced fungal genomes, to obtain new enzymes of interest. Then, the catalytic performance, selectivity and/or stability of the best enzyme candidates were adapted, when needed, to the required reaction conditions using **protein engineering** tools. Several concepts such as substrate loading, co-factor addition, biocatalyst stability and downstream processing, among others, were also considered to further **optimize** the enzymatic reactions. Finally, life-cycle assessment (**LCA**) analyses of the new enzymatic processes, compared with chemical processes for the production of the same or similar compounds, were performed for the final evaluation of their environmental, as well as technical and economic, feasibility. Some examples of the work performed and how it is beyond the state-of-the-art, as demonstrated by different publications and patents, are provided in the next sections.



INDUSTRIAL ENZYMES

Although the selection of new enzymes was largely based on activity detection in fungal cultures, and sequences available in databases and already sequenced genomes, two new genomes representing groups of fungi scarcely investigated for enzymes of interest (the ascomycetes *Lecytophora hoffmanii* and *Kretzschmaria deusta*) were **sequenced** in the course of the project (2,3). Cultures and genomes were screened for new types of unspecific peroxygenases (**UPO**), the main and most promising oxidoreductase type in EnzOx2 (4,5), whose industrial applicability was improved by application of different enzyme engineering methods and structural-functional studies (6-10). **Oxidases** were also largely investigated in the project to catalyze reactions of interest or as a source of the hydrogen peroxide required by UPOs. Efficient in situ generation of H_2O_2 is critical to achieve high turnover numbers with UPOs, an aspect that has been addressed in several EnzOx2 studies (11-13). Aryl-alcohol oxidase (**AAO**) was selected as a model oxidase, and its reaction mechanism with both reducing (benzylic alcohols) and oxidizing (molecular oxygen) substrates was in-depth investigated (14-16) for its application as an enzymatic catalyst. The above oxidoreductase applications include new enzymatic technologies for both **sugar** and **lipid** derived building blocks and other added value compounds.

ENZYMATIC TECHNOLOGY FOR SUGAR DERIVED BUILDING BLOCKS

Concerning the enzymatic production of **sugar** (furfural) based building blocks, the project focused on the enzymatic conversion of HMF to 2,5-furandicarboxylic acid (FDCA), a three-step oxidative process. FDCA has been classified as one of the top 12 value-added chemicals derived from biomass, because it is the renewable precursor for the production of poly (ethylene-2,5-furandicarboxylate) (PEF), the polymer expected to substitute for petroleum-derived poly(ethylene-terephthalate) (PET) plastics in the near future. We optimized the previously described two-enzyme cascade for HMF conversion into FDCA, and proposed a new cascade involving AAO, UPO and galactose oxidase with additional properties of interest (17). Interestingly, a recent study revealed that the inability of AAO to complete HMF conversion into FDCA is, at least partially, due to inhibition of the last oxidation step by the H_2O_2 generated during the two previous oxidation steps and, therefore, can be abolished by the addition of catalase (18).

Moreover, since large-scale production of FDCA-based bioplastics (e.g. by the joint venture of companies BASF and Avantium) will be based on 5-methoxymethylfurfural (MMF) in addition to HMF, the former compound was also included in EnzOx2. A self-sustained three-member enzymatic cascade for FDCA production from MMF was developed (13) and patented (19), where the action of UPO is fueled by hydrogen peroxide provided by both AAO and a second oxidase acting on the methanol release from the demethoxylation of MMF by UPO. Finally, the production of PEF from enzymatically-synthesized FDCA is being evaluated by AIMPLAS and Ava Biochem, compared with the other synthetic routes available.





EnzOx2
Enzymatic oxidation/oxygenation

ENZYMATIC TECHNOLOGY FOR LIPID DERIVED ADDED VALUE COMPOUNDS

For the enzymatic production of **lipid** “sensu lato” added value compounds, the action of UPOs on different compound types has been investigated. Among them, the enzyme from *Marasmius rotula* catalyzes unique reactions on fatty acids including terminal/subterminal hydroxylation and chain shortening. The latter has been fully investigated from enzymatic and mechanistic points of view (20) and a patent has been deposited for the controlled one-carbon shortening of fatty acids (21), a reaction representing a novel chemistry that may be used in biotechnological applications to obtain tailor-made acids not abundant in nature. Terminal hydroxylation, of interest for homopolyester production and other applications, is being investigated with UPOs.

Among steroid transformations, the enzyme from the basidiomycete *Marasmius rotula* (and a second *Marasmius* species) was also able to remove the side chain of cortisone for production of APIs (22) in a reaction reminiscent of the chain shortening mentioned above. A second steroid transformation of pharmaceutical interest was demonstrated for the UPO of the ascomycete *Chaetomium globosum*, which catalyzes the selective oxygenation of testosterone (23). This reaction, yielding 90% testosterone epoxide (isolated with 96% purity) has been recently up-scaled and positively evaluated for industrial implementation. Two additional reactions of pharmaceutical interest catalyzed by several UPOs are the hydroxylation of stilbene (and several stilbenoids) for the selective synthesis of resveratrol analogues (24) and the hydroxylation of propranolol (25,26).

Challenging selective oxyfunctionalization of four model terpenes has been actively investigated by the above and other UPOs, although the developed bioprocesses did not fulfil yet the feasibility requirements for their industrial implementation in the flavour & fragrance industry. Moreover, selective synthesis of 4-hydroxyisophorone and 4-ketoisophorone, of interest for both pharmaceutical and flavour & fragrance sectors, has been reported with UPOs (27) and protected by a patent application (28).

INDUSTRIAL IMPACT

The industrial impact of the above enzymatic transformations is related to: **i)** the use of environmentally-friendly enzymatic technologies in the manufacture of bio-based bulk chemicals such as plastic building blocks; and **ii)** the selectivity of UPOs catalyzing specific oxygenation reactions of pharmaceuticals, and other speciality chemicals, that are difficult or very complicated (expensive) to be obtained using chemical methods. Several publications on their environmental feasibility have been already produced in EnzOx2 (29-31).



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